


Defining centric diatoms of most relevant phytoplankton functional groups in deep karst lakes

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Abstract Phytoplankton in karst lakes is characterized by co-occurrence of chrysophytes (Chrysophyceae), dinoflagellates (Dinophyceae), and diatoms (Bacillariophyta) as the most diverse and abundant group. Using Reynolds functional approach in ecological phytoplankton investigations makes karstic lakes comparable and facilitates interpretation of their responses to changing environmental conditions. Accurate taxonomic identification to species level,

based on precise resolution of specific characteristics by electron microscopy, is essential in order to correctly associate species into coda. This paper defines the most abundant centric diatoms and coexisting species in the phytoplankton of karst lakes in Croatia as a contribution to better description of phytoplankton functional groups dominated by centric diatoms. Several representatives for diatom-based Reynolds functional groups of four lakes in Croatia were described in this paper: *Pantocsekiella costei* and *Stephanocostis chantaica* for group A, *Pantocsekiella ocellata* and *Cyclotella distinguenda* for group B, and *Stephanodiscus neoastraea* for group C, together with their ecological preferences which clearly correspond to pertaining functional classification. Selected and coexisting functional groups define natural, oligo- to mesotrophic karst deep lake systems.

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Introduction

Phytoplankton comprises extremely diverse, polyphyletic group of organisms, demonstrating a wide array of morphological, physiological, and behavioral traits (Litchman & Klausmeier, 2008). Due to the high phenotypic variability, phytoplankton species represent distinct ecological entities that respond to resource availability, environmental stimuli, predators, and intra- and inter-species interactions. Such

high phytoplankton diversity accounts for different functions within the freshwater ecosystems, and thus its understanding in space and time is highly relevant and timely issue. Correct species identification is important because in many cases taxonomically similar planktonic species do not represent ecological equivalents. Changes in the species concept over the past few decades (Salmaso et al., 2014) resulted in many new species descriptions, creating taxonomic uncertainties and confusion. In addition, limnological studies have demonstrated that similar taxonomic groups can co-occur in different phytoplankton assemblages with same ecological preferences. These difficulties in species identification and their ecology have led to a development of new approaches, like the creation of phytoplankton functional groups. The main aim of the new functional approach in studying phytoplankton assemblages was to improve the traditional ecological and taxonomical species concept (Salmaso et al., 2012). During the past few decades, three morpho-functional classifications have been described and reported as the most useful to assess phytoplankton functionality and seasonality (Borics et al., 2012; Salmaso et al., 2012); Functional Groups (FG), Morpho-Functional Groups (MFG) and Morphologically Based Functional Groups (MBFG) (Reynolds et al., 2002; Salmaso & Padisák, 2007; Kruk et al., 2010). Reynolds (1980, 1984) proposed one of the first classifications based on the species physiological, morphological, and ecological features, also including the range of conditions within which different species co-occur. Reynolds FGs concept was based on the similarity of the species ecological niche and environmental preferences. Therefore, phytoplankton functional groups can be seen as groups of species with more or less precisely defined requirements for several different combinations of physical, chemical, and biological properties of the lake environment (Padisák et al., 2006). Subsequently, Reynolds et al. (2002) described 31 FGs or “coda” based on a large species database and their own expertise and knowledge. Using Reynolds functional approach makes environmental investigations comparable and facilitates evaluation of freshwater bodies’ responses to changing environmental conditions. During the past decade, data have been complemented to more than 40 coda (Padisák et al., 2009) and successfully applied to a wide range of different ecosystems: tropical coastal lagoons (Alves-de-Souza et al., 2006), tropical

estuaries (Costa et al., 2009), subarctic lakes (Forström, 2006), barrage lakes (Nabout & Nogueira, 2007), subtropical lakes (Kruk et al., 2002), subalpine lakes (Morabito et al., 2003), temporary limestone lakes (Pereira et al., 2011), shallow (Paształeniec & Poniewozik, 2010) and deep temperate zone lakes (Souza et al., 2008; Soylu & Gönülol, 2010), large rivers (Borics et al., 2007; Salmaso & Braioni, 2007; Abonyi et al., 2012, 2014; Stanković et al., 2012), and karst lakes (Gligora et al., 2007; Žutinić et al., 2014; Gligora Udovič et al., 2015).

In the array of freshwater ecosystems, karst lakes are unique by their geological, physical, and chemical properties (e.g., Mikac et al., 2011). In most oligo- and mesotrophic karst lakes, phytoplankton is characterized by the presence of different algal groups like chrysophytes (Chrysophyceae) and dinoflagellates (Dinophyceae), and diatoms (Bacillariophyta) as the most diverse and abundant group (Gligora Udovič et al., 2015). Within diatoms, centric species belonging to Mediophyceae (Medlin & Kaczmarek, 2004) are the most common descriptors of phytoplankton functional assemblages. When information on characters pertaining to populations is essential in addressing particular ecological questions or environmental issues, accurate identification of dominant species in one functional group is highly important. During ecological investigations of karst lakes (Gligora et al., 2007; Gligora Udovič et al., 2011, 2015; Žutinić et al., 2014), we found that the morphology of centric diatoms is extremely variable and that the inadequate taxonomic resolution may often result in incorrect placement of species into coda, or even prevents sorting into coda. Therefore, accurate taxonomic identification to a species level, based on a precise resolution of specific characteristics by electron microscopy, is essential in order to correctly associate species into coda. This paper defines the most abundant centric diatoms and coexisting species in the phytoplankton of karst lakes in Croatia as contribution to better description of phytoplankton functional groups dominated by centric diatoms. Therefore, specific aims of this work are as follows: (i) to address, using SEM micrographs, main characteristics necessary to describe each of the centric diatom species within FGs; (ii) to explore main environmental conditions controlling these functional groups and; and (iii) to clarify whether the karst habitat fits into environmental features of a given FG (codon).

Study sites

Croatian karstic lakes, including Vransko, Visovačko, Kozjak, and Prošće, are very heterogeneous by origin and type. The high proportion of carbonate bedrocks in the aquatic systems of the Croatian karst enabled formation of a specific tufa barrages, cascades, and/or sheets (e.g., Primc-Habdića et al., 2001; Pavlović et al., 2010). Tufa can be present in many forms, but probably the most famous are the travertine barriers that shape the waterfalls, like those of Plitvice Lakes and the Krka River (Kralj et al., 2006). Lake Prošće is the upper most of the sixteen cascading lakes forming Plitvice Lakes system with Matica River as an input of dissolved trace elements (e.g., As, Cr, Ni, Cu, Pb), of which concentrations sharply decrease downwards, thus indicating intensive deposition process. Calcium, a major element playing the key role in geochemistry of the Plitvice Lakes, also shows a decrease in the dissolved phase downwards due to intensive calcite precipitation (Miliša et al., 2006; Barešić et al., 2011; Dautović et al., 2013). As opposed, Lake Kozjak is the deepest and largest lake in the Plitvice Lakes system, with a longer water retention time, which ultimately results in a higher algal production in summer and autumn, contrary to spring algal maximum in Lake Prošće (Žutinić et al., 2014). Lake Visovačko, a lentic dilatation of the Krka River, also belongs to a group of karstic barrage lakes (Petrik, 1965; Gligora Udovič et al., 2011), while Vransko is an isolated, deep cryptodepression located on the Cres Island, Adriatic Sea (Petrik, 1965; Bonacci, 1993, 2014). Opposite to Plitvice Lakes, which are situated in continental part of the country with continental climate conditions, lakes Visovačko and Vransko are influenced by the mediterranean climate.

Materials and methods

Phytoplankton samples were collected monthly from April to September 2009 from lakes Visovačko and Kozjak, and 2010 from lakes Vransko and Prošće. Sampling was performed at 5 m intervals in the euphotic zone at the deepest part of each lake. Additional samples were taken in 2015 and used for detailed morphological analyses and comparison of the species. Samples used for water chemistry analysis were taken simultaneously and measured according to

APHA (1995). Values of pH, conductivity, oxygen concentration, and saturation were measured on site with a WTW Multiline P4 portable meter. Secchi disk was used for measurements of water transparency. The euphotic zone (Z_{eu}) was calculated as 2.5 times the Secchi depth (Mischke et al., 2012). The mixing zone (Z_{mix}) was estimated from the temperature and dissolved oxygen profiles. Samples were fixed in 2% final concentration formaldehyde solution. Phytoplankton abundance was determined using inverted microscope (Axiovert 200, Carl Zeiss, Germany) and 50 ml chambers that were settled for 24 h (Utermöhl, 1958). At least 300 cells were counted at 400× magnification, corresponding to a counting error of 10% (Lund et al., 1958). Species cell size was measured with AxioVision LE 4.8.2 software (Carl Zeiss, Germany) using at least 20 individual cells, and cell volumes were calculated after approximation of geometric models following Hillebrand et al. (1999) and Sun & Liu (2003). Biomass was estimated from the product of the population and the mean cell volumes, and density of cells was assumed as 1 g ml^{-1} (Rott, 1981). The identified species were classified into functional groups (FGs) according to Reynolds et al. (2002) and Padisák et al. (2009). Biomass (phyla and FGs) and chemistry data were averaged monthly following WISER recommendation on sampling methodology for the water framework directive (Mischke et al., 2012): (i) When $Z_{eu} > Z_{mix}$ data were averaged down to Z_{eu} ; (ii) When $Z_{eu} < Z_{mix}$ data were averaged down to Z_{mix} .

Non-metric multidimensional scaling (nMDS), Principal Component Analysis (PCA), SIMPER analysis, and Canonical analysis of principal coordinates (CAP) were calculated in PRIMER 6.1.10 (Clarke & Gorley, 2006). Phytoplankton biomass was transformed using the logarithm function prior to statistical analyses.

For examination of *Pantocsekiella* organic material, the formaldehyde preserved water sample was directly filtered on $3 \mu\text{m}$ Nucleopore polycarbonate filter (Nucleopore, Pleasanton, CA) and rinsed with distilled water. After dehydration in a series of ethanol solutions (25, 35, 50, 75, 80, 90%), the sample was prepared with distilled water and absolute ethanol, finishing with three rinses of 100% ethanol. For the drying method, the hexamethyldisilazane (HMDS) treatment was used (Bray et al., 1993). The sample was rinsed in a series of 100% ethanol: HMDS

solutions (3:1, 1:1, 1:3), finishing with three changes of 100% HMDS, with a minimum of 5 min treatment at each step, allowing the last HMDS rinse to evaporate slowly at room temperature. For preparation of cleaned diatom frustules, subsamples were first washed with formaldehyde and subsequently treated with hydrochloric acid (HCl) to remove carbonates, sulphuric acid (H₂SO₄) and sodium nitrate (NaNO₃) to clean the organic matter (2 ml of sample, 1:1, 3:1, 0, 5 g, respectively). Subsamples for scanning electron microscopy (SEM) were filtered onto membrane filters (Whatman Nucleopore 0.3 µm) and attached to aluminum stubs after air-drying. Stubs were sputter-coated with 30 nm of gold (Scancoat, Edwards Ltd., UK). Scanning electron microscopy was performed with a FEG Tescan MIRA3 (TESCAN, Czech Republic). Diatom species identification followed Krammer & Lange-Bertalot (1991), Håkansson (2002), Houk et al. (2010), Kiss et al. (2012), and Cvetkoska et al. (2014). Chrysophyte and dinoflagellate species identification followed Huber-Pestalozzi (1941), Taylor (1987), Popovský & Pfister (1990), John et al. (2002, 2011) and Hansen & Flaim (2007).

Results

Environmental parameters

We studied four different karst lakes: Vransko (Cres Island), Kozjak and Prošće (Plitvice Lakes National Park), and Visovačko (Krka National Park) (Fig. 1). Extended details of the lakes' physical and hydrological features are provided in Gligora Udovič et al. (2015). The main properties of each investigated lake (altitude, size, depth, geographical location) are presented in Table 1, while the physical and chemical measurements of their euphotic and/or mixing zones are listed in Table 2. Two groups were distinguished from the temperature profiles. The group comprising lakes Vransko and Visovačko was characterized by higher temperature minima (13.9 and 11.3°C, respectively) and maxima (20.0 and 22.1°C, respectively), while Kozjak and Prošće group had overall lower temperatures (Table 2) and mean values (12.9 and 12.3, respectively). Variations in pH were low in all lakes, ranging between 7.7 and 8.4 throughout the

investigated period. The lowest alkalinity was measured at Lake Vransko, with a mean value below 120 mg l⁻¹, which classifies it as a moderately hard lake. Alkalinity was much higher in the other lakes, with Lake Prošće having the highest values of 232–237 mg l⁻¹, which classifies it as a very hard-type lake (Durfor & Becker, 1964). Electrical conductivity of the lakes was relatively high, ranging from 425 to 465 µS cm⁻¹, except for Lake Kozjak, where the lowest values and widest ecological valence were detected (368–415 µS cm⁻¹). The mean dissolved oxygen (DO) profile of all lakes showed high saturation level of the euphotic layer throughout the investigated period, while the chemical oxygen demand remained very low with the highest and lowest concentrations of DO noted in Lake Kozjak (Table 2). The concentrations of inorganic nitrogen compounds (NH₄⁺ and NO₂⁻) showed only slight variations in all lakes throughout the investigated period. Ammonia (NH₄⁺) concentrations ranged from 1.0 to 35.0 µg l⁻¹ for Lake Visovačko and Lake Prošće, respectively. The NO₂⁻ concentration was from below the detection limit in Lake Vransko, up to 7.8 µg l⁻¹ in Lake Kozjak. Only the nitrates (NO₃⁻) showed greater variation among the lakes; from below the detection limit to 664 µg l⁻¹ (Lakes Vransko and Kozjak, respectively). Total nitrogen (TN) concentration also varied greatly from 155 (Lake Visovačko) to 805 µg l⁻¹ (Lake Prošće). The mean soluble reactive phosphorus (SRP) and total phosphorus (TP) concentrations remained low in all the lakes (3.2–3.8 and 5.3–14.6 µg l⁻¹, respectively), with exception of Lake Kozjak (0.8–79.8 µg l⁻¹ SRP and 19.6–58.2 µg l⁻¹ TP) (Table 2).

Eight environmental parameters for each lake were used for the principal component analysis (Fig. 2). First two axes accounted for 62.3% of the total variance, with eigenvalues of 3.49 and 1.49 for PCA axis 1 and PCA axis 2, respectively, comprising values for 5 most significant variables. TN and alkalinity were the most important parameters for the PCA axis 1 (intra-set correlations: -0.429 and -0.419, respectively). PCA axis 2 was described by SRP, TP, and DO (intra-set correlations: 0.562, 0.425 and -0.374, respectively). Samples were grouped spatially according to the lake representation. Lake Vransko samples were mainly singled out according to pH and temperature, while samples from Lake Visovačko were described by conductivity and

Fig. 1 Map of the investigated lakes. 1 Vransko, 2 Kozjak, 3 Prošće, 4 Visovačko

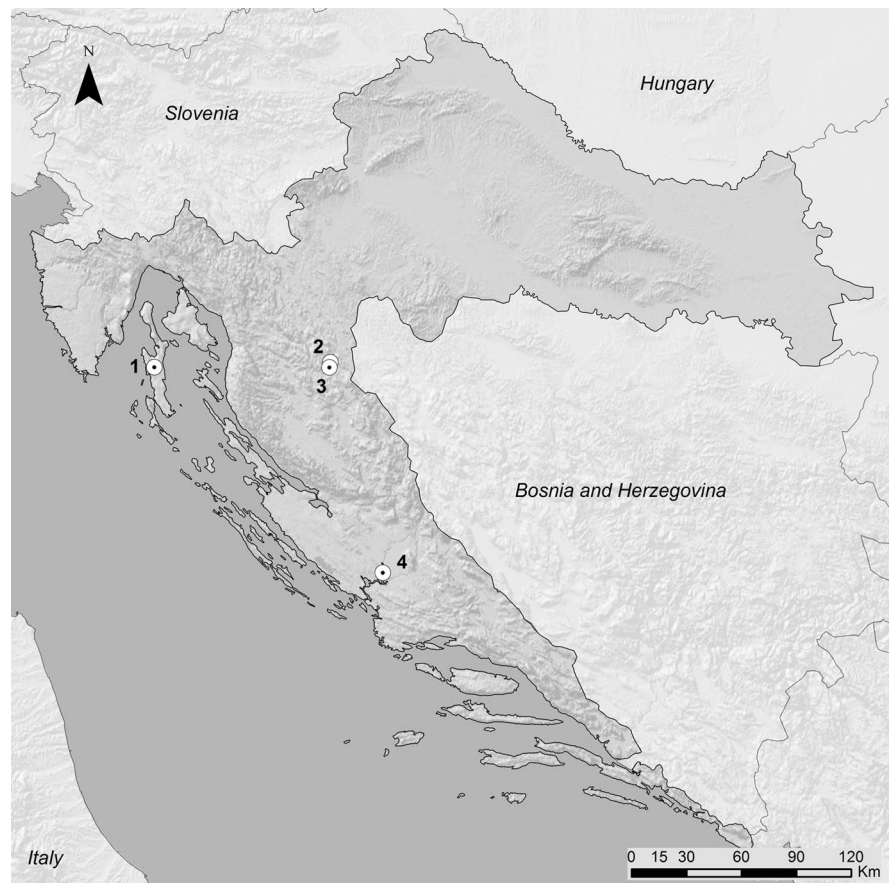


Table 1 Location and physical properties of the investigated lakes

Lake ^(no. on the map)	Elevation (a.s.l.) (m)	Surface area (km ²)	Volume (m ³)	Max depth (m)	Geographic coordinates
Vransko ⁽¹⁾	13	5.75	220.3×10^6	74.5	44°51'13"N, 14°23'23"E
Kozjak ⁽²⁾	535	0.82	12.71×10^6	48.0	44°53'27"N, 15°36'16"E
Prošće ⁽³⁾	636	0.70	7.67×10^6	38.5	44°52'06"N, 15°35'51"E
Visovačko ⁽⁴⁾	47 (Skradinski buk)	5.72	103×10^6	30.0	43°51'33"N, 15°58'37"E

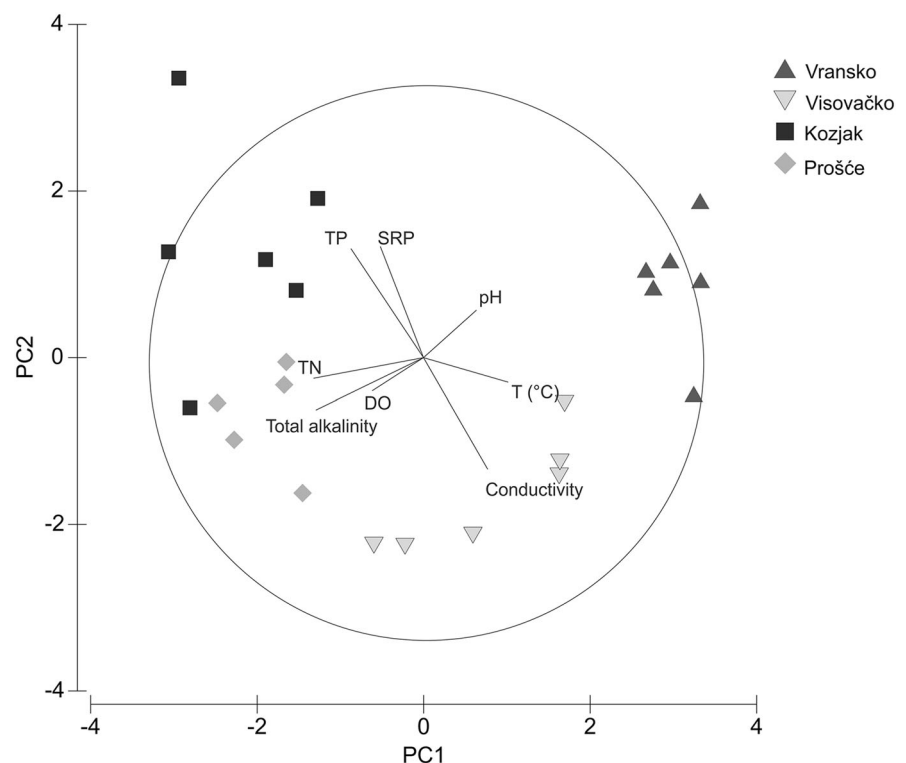
temperature. The most important parameters for lakes Kozjak and Prošće were nutrients (TP, TN, SRP), DO and total alkalinity.

CAP results calculated from the phytoplankton FG biomass values and the environmental variables are shown in Fig. 3. In this case, both the eigenvalues (correlation coefficients) for the first two axes (0.9819 and 0.8619, respectively) and the squared environment-species correlations (0.9641 and 0.7429, respectively) were significantly positive. Permutation test showed that the ordination of both axes was statistically significant ($P < 0.001$). The first CAP axis was

strongly positively correlated with $\text{NH}_4\text{-N}$, pH, and TN (canonical eigenvectors: 0.482, 0.468, and 0.453, respectively) and negatively with temperature (canonical eigenvector -0.400), whereas the second CAP axis was mainly defined by total alkalinity and conductivity (canonical eigenvectors: 0.692 and 0.427, respectively). According to the results, three groupings were observed (with a selected threshold of 40% similarity). One group, comprising samples from Lake Vransko with an outlier from Lake Visovačko, positioned at the negative end of CAP2 axis and was constrained by conductivity and pH. Samples from

Table 2 Ranges of environmental parameters in the euphotic zone (Z_{eu}) or mixing zone (Z_{mix}) of the investigated lakes

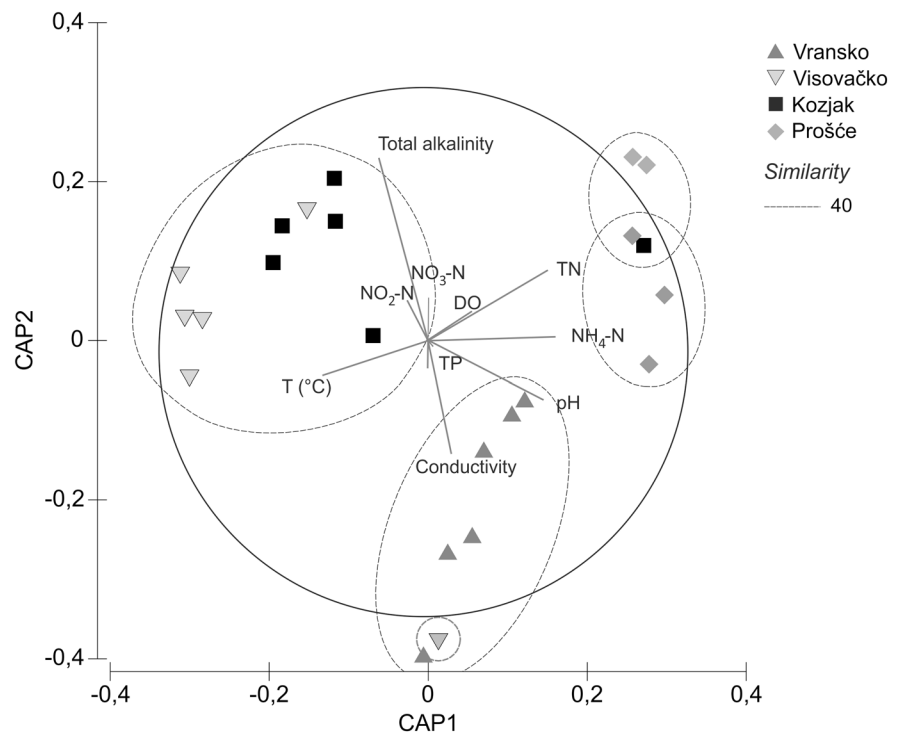
	Vransko			Visovacko			Kozjak			Prošće		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
Temp (°C)	13.9	21.0	18.1	11.3	22.1	17.4	5.9	16.5	12.9	9.4	15.6	12.3
Secchi (m)	10.5	16	13.25	3	7	4.6	8	14.5	10.5	4.5	7.5	5.75
pH	8.2	8.4	8.3	7.8	8.0	7.9	7.7	8.4	7.9	8.0	8.2	8.1
Conductivity ($\mu\text{S cm}^{-1}$)	429.3	442.2	436.5	446.0	465.0	453.0	368.9	415.0	386.4	425.6	454.5	433.9
Alkalinity ($\text{mg CaCO}_3\text{l}^{-1}$)	112.4	118.7	115.9	184.0	207.0	194.5	208.7	224.0	217.3	232.8	237.3	234.9
DO (mg l^{-1})	10.2	11.1	10.7	9.6	11.4	10.7	9.3	13.2	11.5	10.3	12.1	11.1
Saturation (%)	106.4	121.1	112.1	104.2	130.7	110.9	88.4	138.2	114.0	96.9	117.3	103.4
COD (mg l^{-1})	1.0	1.5	1.2	0.3	1.2	0.7	0.3	2.2	1.1	0.8	1.2	1.0
$\text{NH}_4\text{-N}$ (NH_4^+ $\mu\text{g l}^{-1}$)	2.0	10.5	3.4	1.0	13.0	6.7	–	–	–	15.0	35.0	24.1
$\text{NO}_2\text{-N}$ (NO_2^- $\mu\text{g l}^{-1}$)	<1.0	1.0	–	1.0	3.0	1.8	0.6	7.8	2.6	1.3	2.8	2.2
$\text{NO}_3\text{-N}$ (NO_3^- $\mu\text{g l}^{-1}$)	<10.0	24.0	–	94.0	350.0	206.3	382.3	664.2	518.8	527.5	617.5	567.1
TN ($\mu\text{g l}^{-1}$)	<270.0	400.0	–	155.0	422.0	252.0	392.1	667.3	523.3	700.0	805.0	748.2
SRP ($\mu\text{g l}^{-1}$)	1.0	12.1	3.7	1.0	7.0	3.8	0.8	79.8	22.8	1.0	7.9	3.2
TP ($\mu\text{g l}^{-1}$)	2.0	34.9	8.8	1.0	9.0	5.3	19.6	58.2	37.8	2.0	26.5	14.6

Fig. 2 PCA ordination diagram showing the variance of the eight environmental parameters measured in lakes Vransko, Visovacko, Kozjak, and Prošće during 2009 and 2010

Visovacko and Kozjak formed a separate cluster confined mainly by temperature and total alkalinity at the negative side of CAP1 axis. The third group,

comprising the samples from Lake Prošće, was restrained by TN and $\text{NH}_4\text{-N}$ at the positive side of CAP2 axis.

Fig. 3 Ordination diagram of canonical analysis of principal coordinates (CAP) performed on eight environmental parameters in lakes Vransko, Visovačko, Kozjak, and Prošće during 2009 and 2010



Assemblage analyses

The nMDS analysis based on species biomass (Fig. 4) showed a clear separation between sampling sites and pointed out descriptor species for centric diatom-dominated functional groups (Fig. 5, 6, 7, 8). The phytoplankton community of Lake Vransko was characterized by a small (5–10 μm) centric diatom *Pantocsekiella costei* (Druart et F. Straub) Kiss & Ács (Fig. 9a–d) as a descriptor species of codon A. This species was constrained by the pH and conductivity, ranging between 8.2–8.4 and 429–442 $\mu\text{S cm}^{-1}$, respectively (Table 2). *Pantocsekiella costei* was the dominant species in the phytoplankton assemblages of Vransko Lake throughout the entire study period, reaching maximum biomass of 0.10 mg l^{-1} in September 2010. *Pantocsekiella ocellata* (Pantocsek) Kiss & Ács (Fig. 10a–e), a descriptor species for codon B, dominated the phytoplankton communities in Lake Visovačko over the entire investigated period (Table 3), reaching a maximum biomass of 1.02 mg l^{-1} in June 2009. The species occurred within alkalinity values of 184–207 mg l^{-1} and temperature range of 11–22 $^{\circ}\text{C}$. Interestingly, *P. ocellata* from Lake Visovačko was often found

forming chains of variable length (Fig. 10a) with cells interconnected with organic, mucilaginous material extruded from their central part (Fig. 10b). Lakes Vransko and Visovačko were also characterized by a range of other centric diatoms (Table 3). *Stephanodiscus neoastraea* Håkansson & Hickel (Fig. 7a–d), a representative of codon C, was the most dominant species in Lake Prošće from July to September 2010, with a maximum biomass in July 2010 (1.69 mg l^{-1}), co-occurring with codon B representatives *Cyclotella distinguenda* Hustedt (Fig. 8a, c, e) (max biomass of 0.34 mg l^{-1} , in September 2010) and *Cyclotella plitvicensis* Hustedt (Fig. 8b, d, f; max biomass of 0.14 mg l^{-1} , in June 2010). For the entire study period, *C. distinguenda* was characteristic for the phytoplankton communities of Lake Kozjak, reaching a maximum biomass of 0.03 mg l^{-1} in July 2009. Both lakes Kozjak and Prošće showed much higher TN and TP concentrations and much lower temperature minima and maxima compared to lakes Vransko and Visovačko (Table 2). *Stephanocostis chantaica* Genkal & Kuzmina, a descriptor species for codon A, and *Lindavia radiosa* (Grunow) De Toni & Forti from codon B were also present in Lake Kozjak, but both were represented with low biomass. Diversity

Fig. 4 Ordination diagram of non-metric multidimensional scaling (nMDS) of phytoplankton species biomass with vector projections of dominant centric diatoms

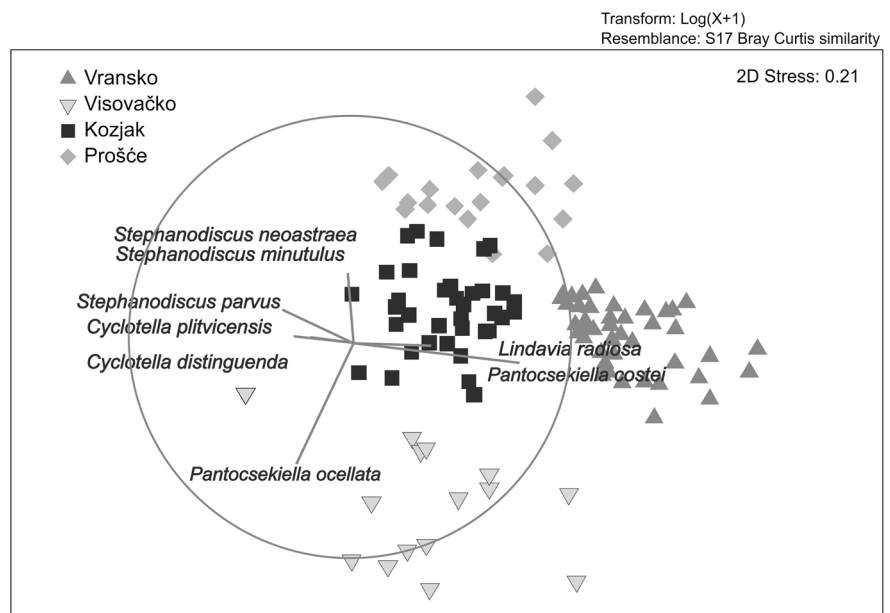
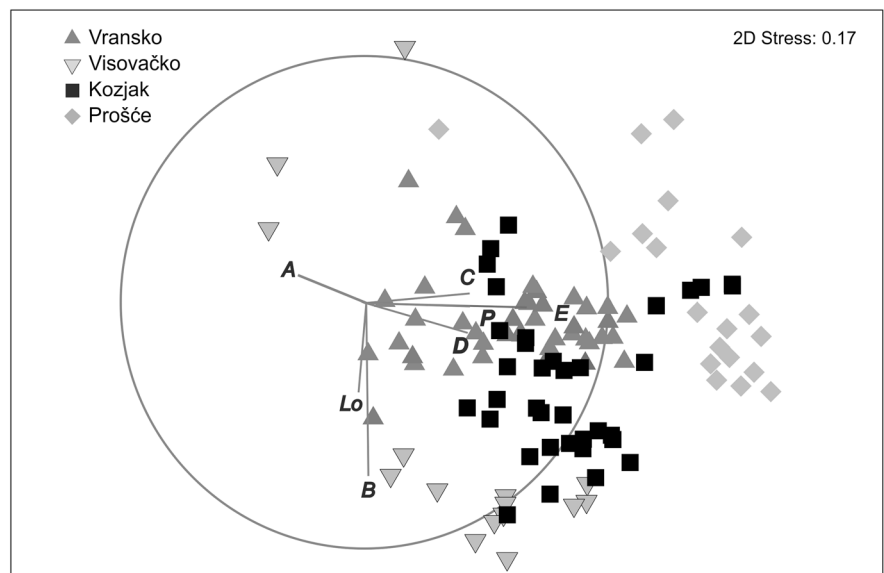


Fig. 5 Ordination diagram of non-metric multidimensional scaling (nMDS) of phytoplankton biomass of functional groups with vector projections of dominant Reynolds functional groups (FG)

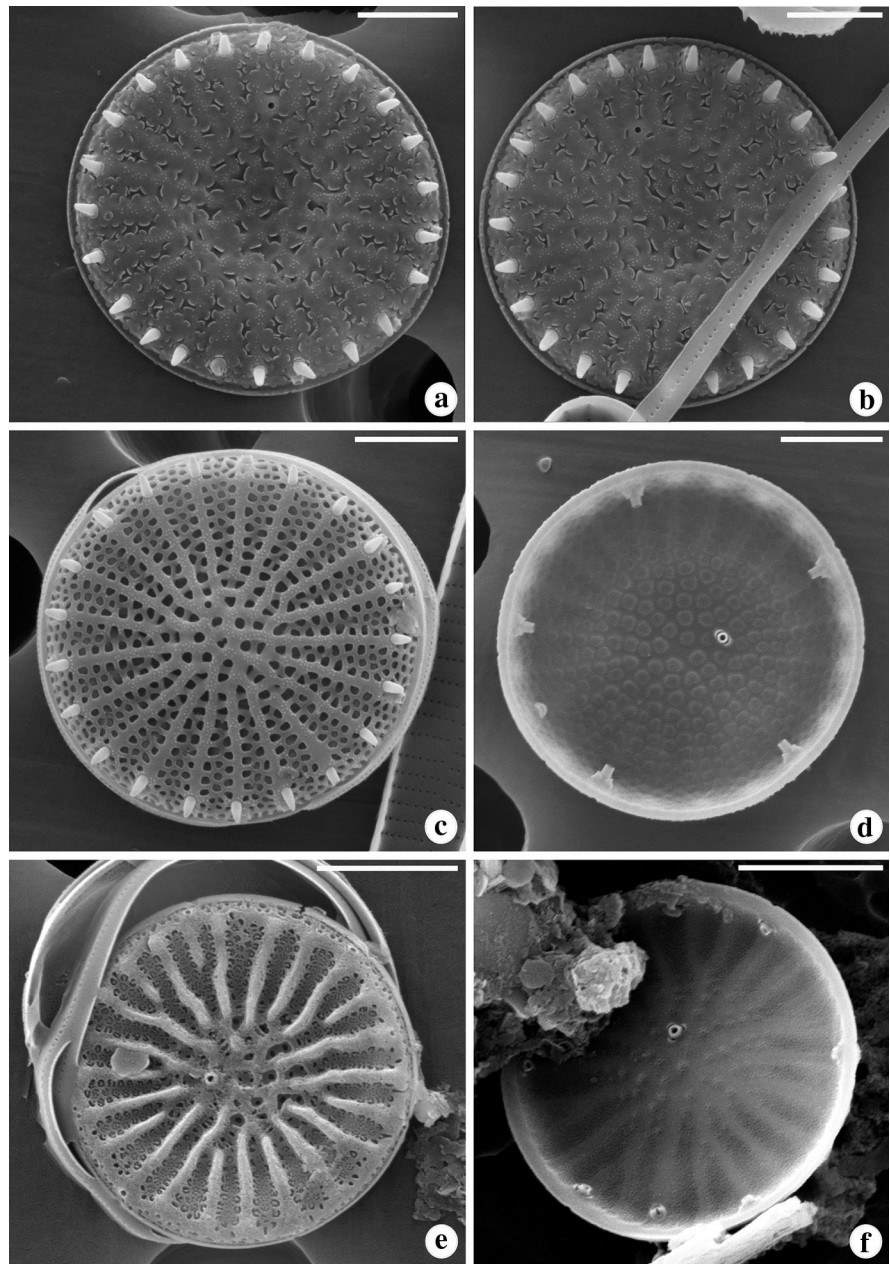


and distribution of diatom species identified in lakes Vransko, Visovačko, Kozjak, and Prošće are presented in Table 3.

Relative contribution (in %) of each FG to average resemblances between pairs of sample groups revealed dominance of different FGs within the plankton assemblages of the lakes (Primer, Simper-test, Table 4). Apart from codon A, Lake Vransko was represented by coda **L_O** and **E** with *Parvodinium inconspicuum* (Lemmermann) S.Carty (Figs. 11, 12)

and *Dinobryon sociale* (Ehrenberg) Ehrenberg/*D. divergens* O. E. Imhof (Figs. 15, 16) as the main descriptive species, respectively. The dominant codon **B** from Lake Visovačko, represented by the chain-forming *P. ocellata* (Fig. 10a–e), was accompanied by *P. inconspicuum* and *Peridinium cinctum* (O. F. Müller) Ehrenberg (Figs. 13, 14, 15, 16) that belong to group **L_O** (Reynolds et al., 2002). Lake Prošće was characterized by centric diatoms from coda **C** and **B**, co-occurring with the following

Fig. 6 **a, b** *Stephanodiscus parvus* Stoermer & Håkansson. **c**, **d** *Stephanodiscus minutulus* (Kützing) Cleve & Möller. **e, f** *Stephanocostis chantaica* Genkal & Kuzmina. **a–c, e** external valve views. **d, f** internal valve views. **a–d** Lake Prošće. **e, f** Lake Vransko. Scale bars 2 μm in (**a–f**)



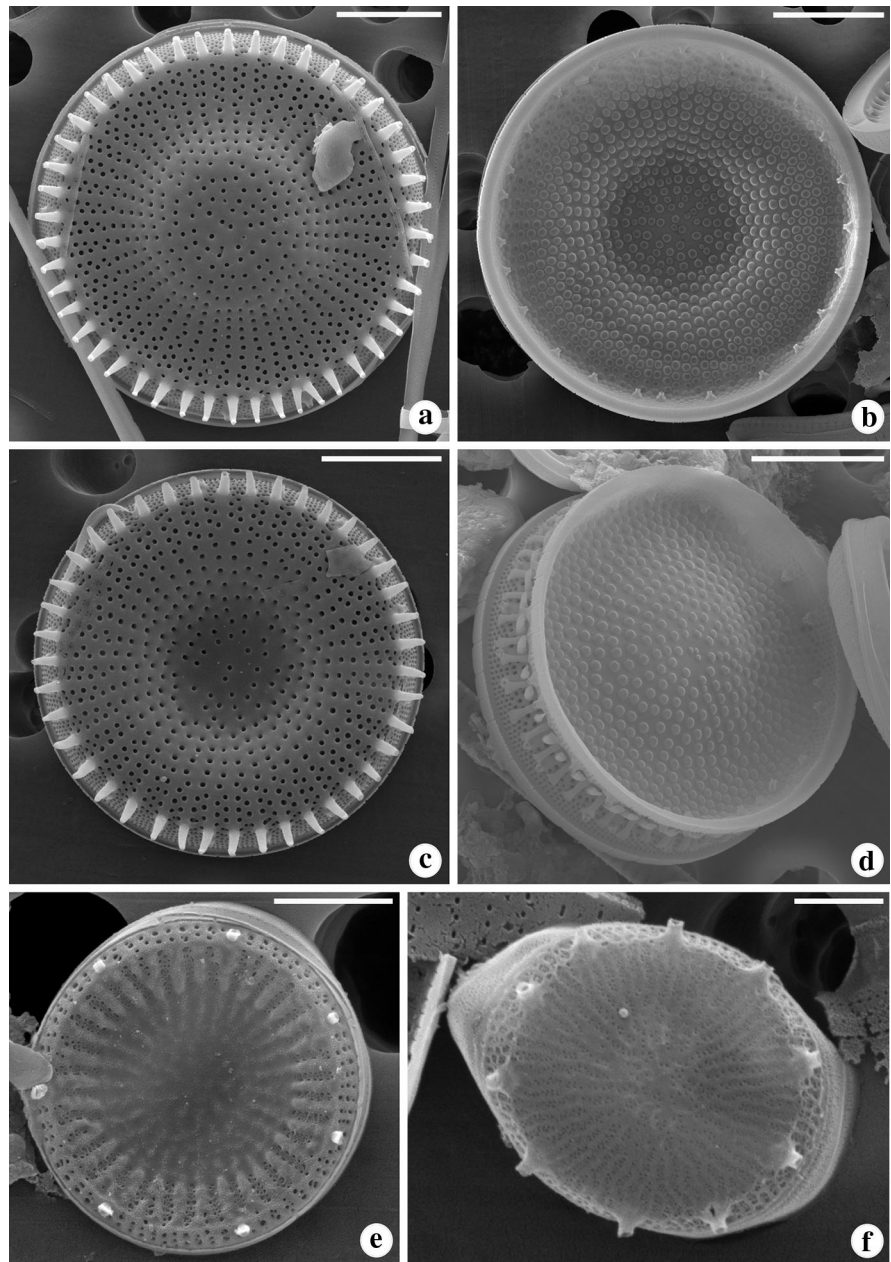
species: *Dinobryon divergens*/*D. sociale*/*D. cylindricum* O. E. Imhof (Fig. 17), *Ulnaria* sp. Ehrenberg, *Fragilaria crotonensis* Kitton, and *Asterionella formosa* Hassall from coda **E**, **P**, and **D**, respectively. Lake Kozjak was co-dominated by group **B** and **E** species (*Dinobryon divergens*/*D. sociale*/*D. cylindricum*), co-occurring with several descriptors from coda **D** and **L_O** (*Ulnaria acus* (Kützing) Aboal and *P. inconspicuum*/*P. cinctum*, respectively).

Discussion

Environmental parameters

All investigated lakes Vransko, Visovačko, Prošće, and Kozjak have a naturally high content of bicarbonates derived from surface, and ground water inputs from the drainage basin are distinguished by having alkaline waters and can be classified as hard karst type

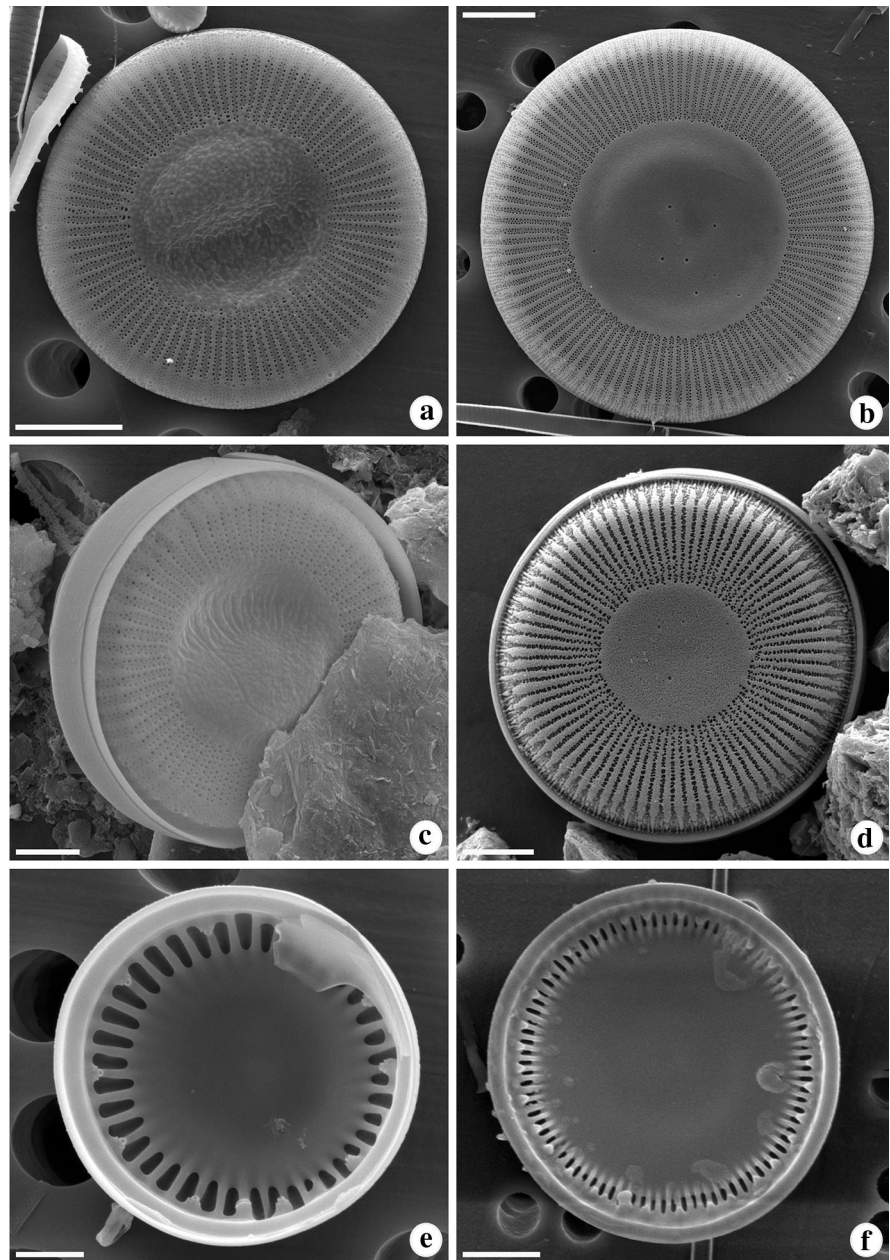
Fig. 7 **a–d** *Stephanodiscus neoastraea* Håkansson & Hickel. **e** *Spicaticribra* sp. **f** *Thalassiosira pseudonana* Hasle & Heimdal. **a, c, e, f** external valve views. **b, d** internal valve views. **a, c** Lake Prošće. **b, d** Lake Vransko. **e, f** Lake Visovačko. Scale bars 5 μm in (**a–e**); 2 μm in (**e**); 1 μm in (**f**)



waters (Vurnek et al., 2010). However, an altitudinal decrease of alkalinity is evident, as also noted by Iveković (1958) and Miliša et al. (2006), presumably as a consequence of intense calcite precipitation (Miliša et al., 2006; Vurnek et al., 2010). Total phosphorus concentrations in these nonpolluted investigated lakes extend over a wide range depending on the productivity of the system. The lowest and the highest TP values were noted in the euphotic zone

during summer stratification, indicating both the bioavailable P and its consumption by the phytoplankton. Mean TP concentrations in lakes Visovačko, Kozjak, and Prošće indicated a slight shift toward a good status as defined by the Regulation on Quality Standards for Water (National Gazette, 73/13; National Gazette, 78/15; National Gazette, 151/14). Soluble reactive phosphorus, as an inorganic P form available for uptake by the phytoplankton (Overbeck,

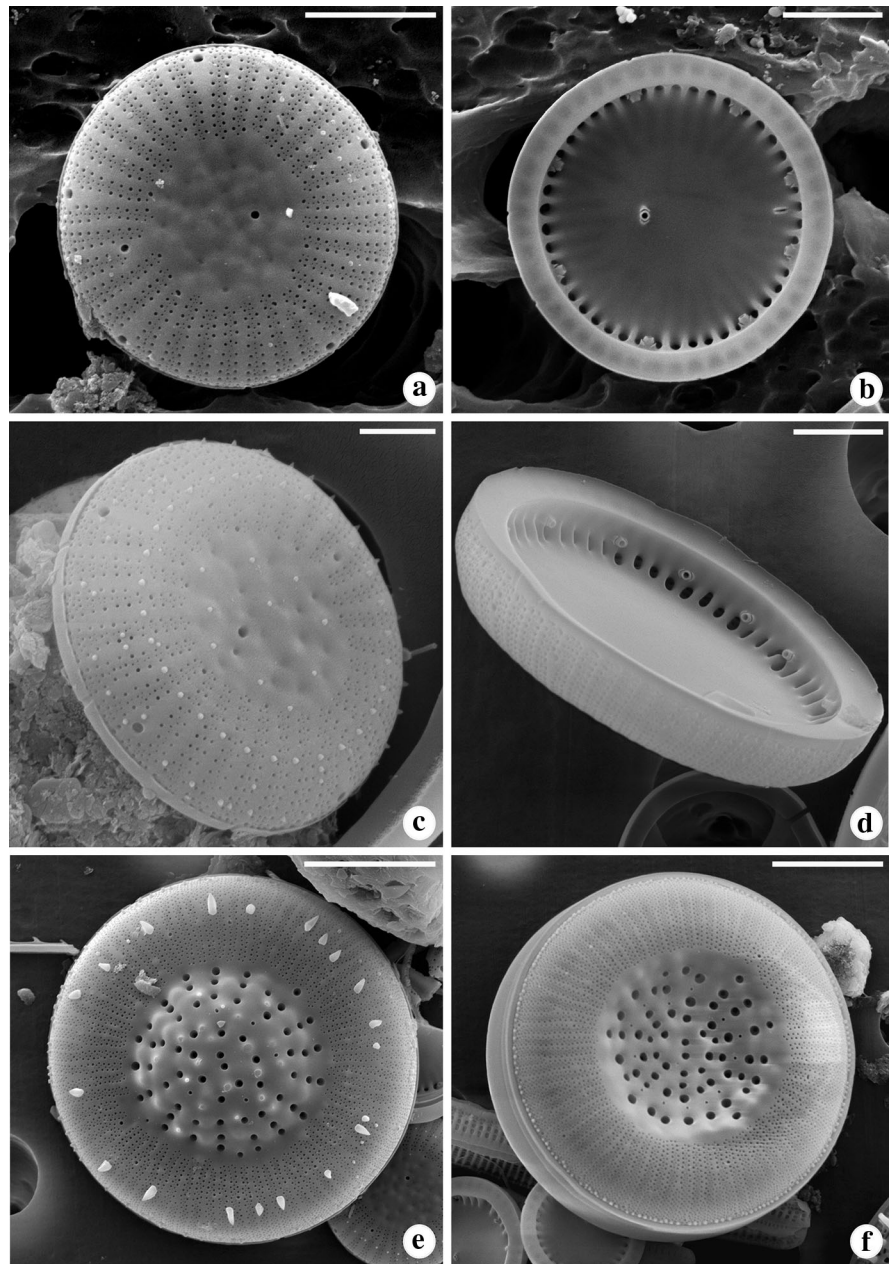
Fig. 8 **a, c, e** *Cyclotella distinguenda* Hustedt. **b, d, f** *Cyclotella plitvicensis* Hustedt. **a–d** External valve views. **e, f** internal valve views. **a–d, f** Lake Prošće. **e** Lake Visovačko. Scale bars 5 μm in (**a, b, d, f**); 2 μm in (**c, e**)



1991), showed low values in all lakes, sometimes even below the range of detection as also noted in previous studies (Horvatinčić et al., 2006; Matoničkin Kepčija et al., 2006). The lowest recorded P concentrations usually occur during the summer period, when the productivity and P bioaccumulation rate are the highest. Moreover, increased pH values in lakes enhance the formation of calcium carbonate, thus coprecipitating available phosphate along with

carbonates (Otsuki & Wetzel, 1972) and removing it from the water column. Calcareous hardwater lakes situated on limestone and dolomite waterbed are distinguished by having a strong carbonate buffering capacity with a relatively high pH values of water, as a consequence of dissolution of the substrate (Wetzel, 2001; Žutinić, 2014). Even slight changes in pH strongly affect phytoplankton assemblages, therefore higher pH noted in lakes during spring is a clear

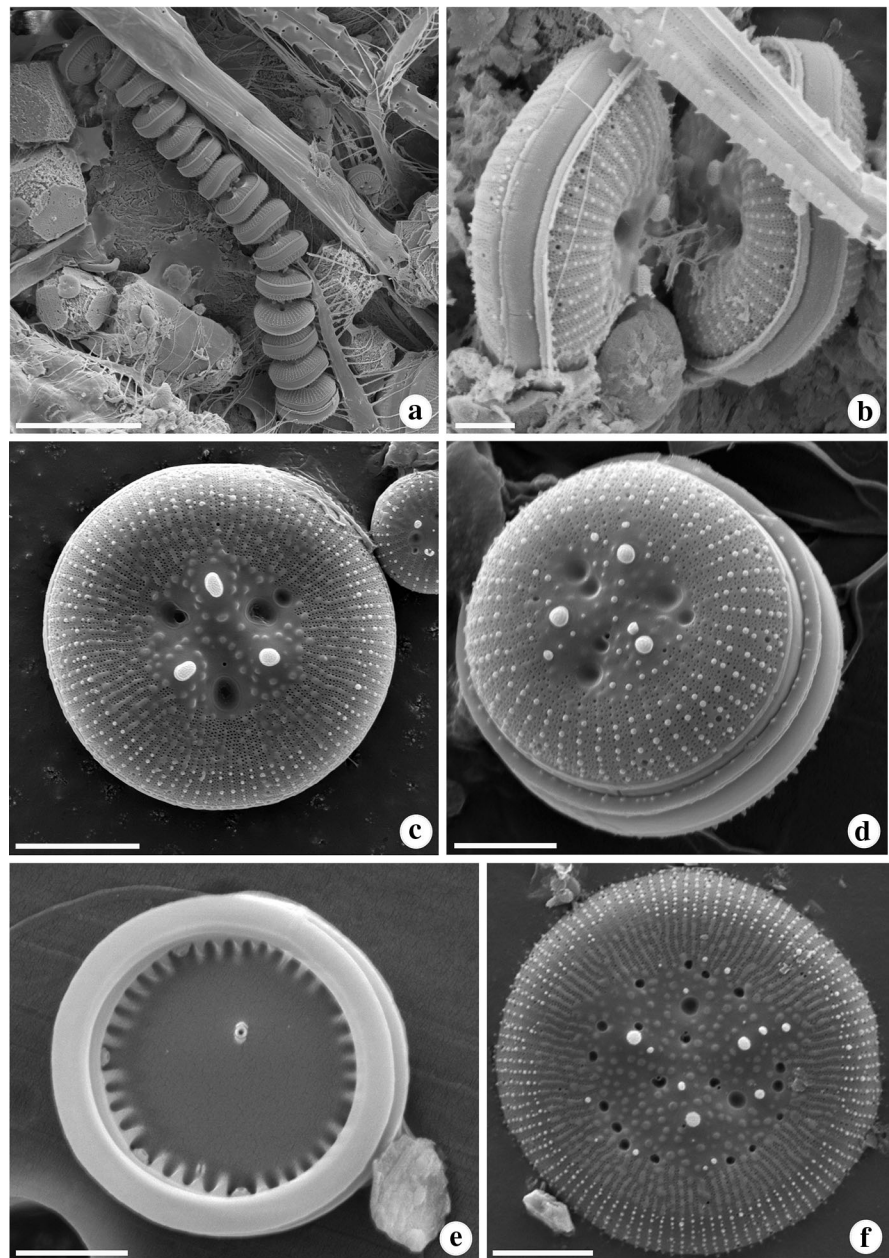
Fig. 9 **a–d.** *Pantocsekiella costei* (Druart & Straub) Kiss & Ács. **e, f** *Lindavia radiosa* (Grunow) De Toni & Forti. **a, c, e, f** External valve views. **b, d** internal valve views. **a–d** Lake Vransko. Scale bars 5 μm in (**e, f**); 2 μm in (**a, b, d**); 1 μm in (**c**)



indication of plankton activity (Habdija et al., 2009). The phytoplankton community in Lake Vransko has access to less nutrients and bicarbonates, thus differing from other lakes and representing almost reference community for oligotrophic karst lakes exclusively described by centric diatoms, chrysophytes and dinoflagellates. The impact of direct precipitation input on the hydrological system of the lakes is visible through differences in conductivity. In barrage lake

systems it is an indicator of calcium carbonate precipitation on tufa barriers between the systems (Horvatinčić et al., 2006; Biondić et al., 2010; Vurnek et al., 2010). The majority of the nitrogen in lakes usually comes from direct terrestrial runoff, but since it can be rapidly oxygenized, concentration of NO_2^- is usually very low, as was the case in the investigated karst lakes. Contrary to NO_2^- values, NO_3^- levels can oscillate greatly depending on the season and

Fig. 10 **a–e** *Pantocsekiella ocellata* (Pantocsek) Kiss & Ács. **f** *Pantocsekiella polymorpha* (Meyer & Håkansson) Kiss & Ács. **a–d, f** External valve views. **e** Internal valve view. **a** *P. ocellata* chain of 15 cells. **b** Two cells of *P. ocellata* with extruded organic material in the central part of the frustules. **a–d, f** Lake Visovačko. **e** Lake Vransko. Scale bars 20 μm in (**a**); 5 μm in (**c, f**); 2 μm in (**b, e**)



surroundings. They were higher in lakes Prošće and Kozjak (Table 2), probably as a result of groundwater percolating through the humus layer of the wooded environment and transporting nutrient to the surface spring waters from which they are flushed in the Plitvice Lakes system (Žutinić, 2014). Increased N deposition has a particularly strong impact on karst lakes (Camacho et al., 2003; Gusev, 2008), therefore even small changes in N inputs can induce substantial

changes in productivity and N cycling (Sheibley et al., 2014).

Assemblage analyses

Reynolds FG classification is based on the idea that, in order to be correctly associated into appropriate coda, all specimens from an assemblage should be identified to a species level (Reynolds et al., 2002). Diatom

Table 3 List of centric diatoms (Bacillariophyceae) identified in phytoplankton assemblages of investigated lakes and their assignment to Reynolds functional groups (FG)

Species	Vransko	Visovačko	Kozjak	Prošće	FG
<i>Actinocyclus normanii</i> (Gregory ex Greville) Hustedt		+			D
<i>Pantocsekiella costei</i> (Druart & Straub) Kiss & Ács	+	+	+	+	A
<i>Cyclotella distinguenda</i> Hustedt	+	+	+	+	B
<i>Pantocsekiella ocellata</i> (Pantocsek) Kiss & Ács		+			B
<i>Cyclotella plitvicensis</i> Hustedt	+			+	B
<i>Pantocsekiella polymorpha</i> (Meyer & Håkansson) Kiss & Ács		+			B
<i>Discostella pseudostelligera</i> (Hustedt) Houk & Klee		+			B
<i>Lindavia radiosa</i> (Grunow) De Toni & Forti	+				B
<i>Melosira varians</i> Agardh		+			T _B
<i>Spicaticribra</i> sp.		+			B
<i>Stephanocostis chantaica</i> Genkal & Kuzmina	+				A
<i>Stephanodiscus minutulus</i> (Kützing) Cleve & Möller			+	+	B
<i>Stephanodiscus neostraea</i> Håkansson & Hickel	+		+	+	C
<i>Stephanodiscus parvus</i> Stoermer & Håkansson		+	+	+	C
<i>Thalassiosira pseudonana</i> Hasle & Heimdal		+			B

Table 4 Contribution (in %) of the most representative FGs in the four investigated lakes (Primer, Simper-test)

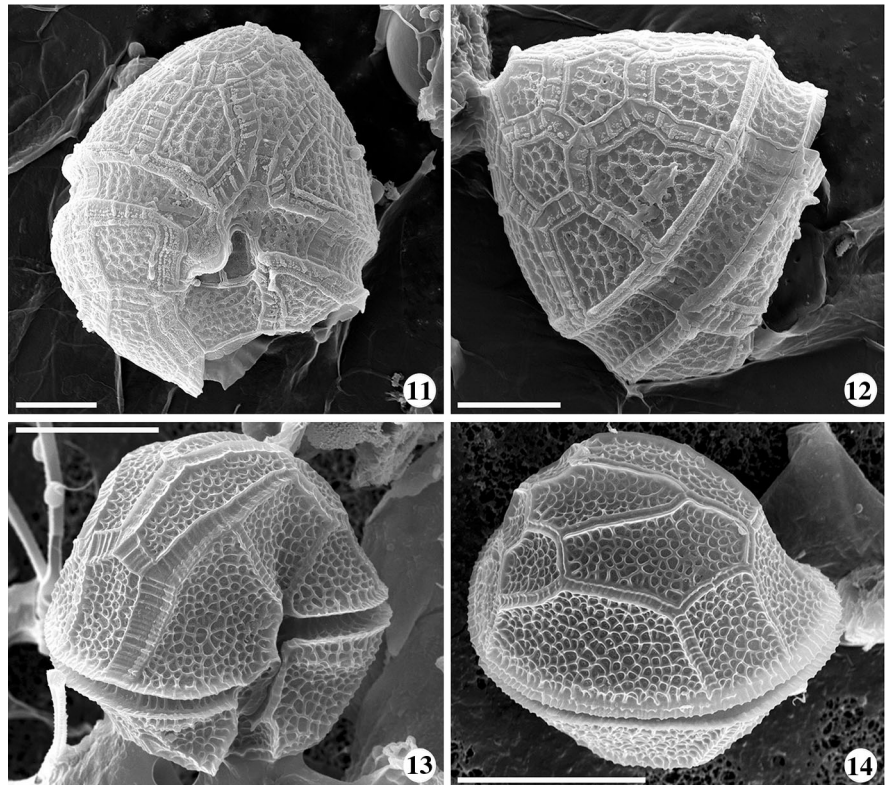
	Vransko	Visovačko	Kozjak	Prošće
A	32.6			
B		71.0	39.9	5.3
C				47.9
D			9.8	3.3
E	37.3		40.3	27.3
L_O	27.5	8.5	6.3	
P				9.2

species identification, specifically from the centric genera like *Cyclotella*, *Stephanodiscus* and *Cyclotella*, has already been qualified as an arduous task, requiring long-term experience in taxonomy in combination with molecular techniques (Cox, 2014). Recent revisions and/or descriptions of new centric diatom genera like *Stephanocostis* (Genkal & Kuzmina, 1985), *Discostella* (Houk & Klee, 2004), *Spicaticribra* (Johansen et al., 2008), *Lindavia* (Nakov et al., 2015) and *Pantocsekiella* (Ács et al., 2016) have increased our knowledge about the diversity of freshwater centric diatoms (Karthick & Kociolek, 2011), but also stressed a further need for detailed phylogenetic analyses of this group of taxa (Kociolek & Khursevich, 2013). In particular, difficulties like barely discernible differences in the morphological

structures under light and scanning electron microscopy (Kling & Håkansson, 1988; Abonyi et al., 2012; Kistenich et al., 2014), interspecific similarity (Sabater & Klee, 1990), and largely unknown species ecology (Sládeček, 1986) greatly account for erroneous or inaccurate species identifications (Håkansson & Kling, 1994) and related misplacements into FGs. Therefore, both taxonomical accuracy and precise functional characterization (Izaguirre et al., 2012; Žutinić et al., 2014) are fundamental for adequate ecological assessments (Cavalcante et al., 2013) and monitoring studies (Holzenhals et al., 2010; Martín & Reyes Fernández, 2012).

Reynolds classification sorts phytoplankton species with similar morphological, physiological and ecological traits into ecological categories—functional groups (FGs) (Reynolds, 1984; Reynolds et al., 2002). Centric diatoms from Reynolds codon A (e.g., *Urosolenia*, *Cyclotella*, *Thalassiosira*) are prominent members in the plankton of many clear, dilute, phosphorus-deficient, high-latitude medium-to-large lakes (Reynolds et al., 2002; Padisák et al., 2009). The diatom community of Lake Vransko, dominated by *P. costei*, and with *Lindavia radiosa* and *S. chantaica* as descriptor species, was restrained by conductivity and pH. Furthermore, physiological properties and ecotype placement (Gligora Udovič et al., 2015) of Lake Vransko is congruent with the

Figs. 11–14 **11, 12** *Parvodinium inconspicuum* (Lemmermann) S.Carty. **13, 14** *Peridinium cinctum* (Müller) Ehrenberg. **11–14** Lake Visovačko. Scale bars 25 μm in (**14**); 20 μm in (**13**); 5 μm in (**11, 12**)

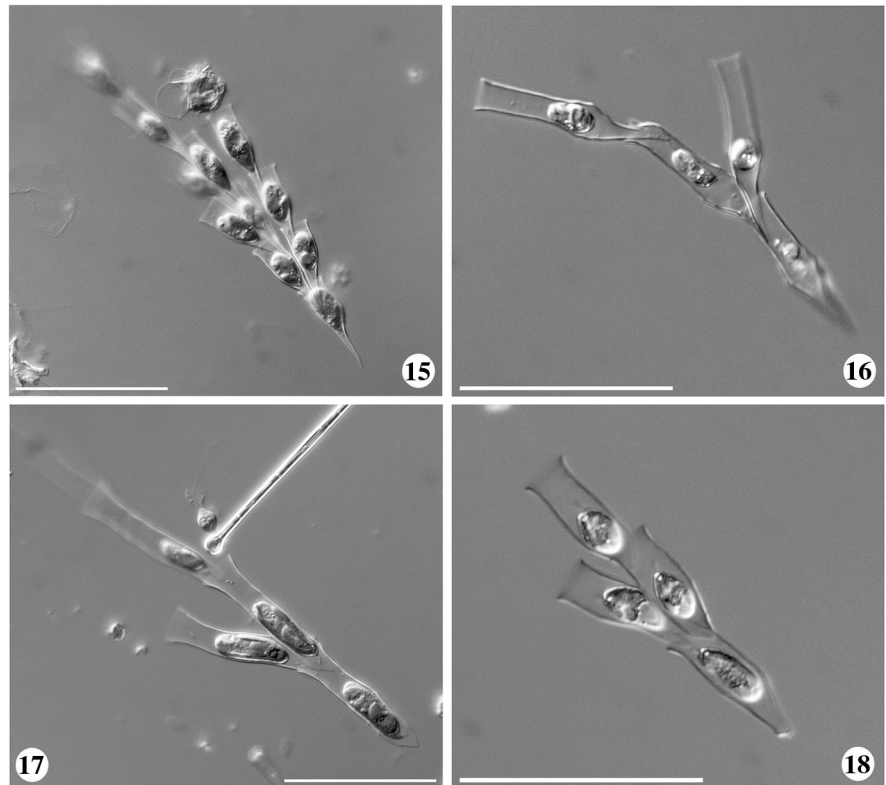


placement of its dominant, typifying species into codon A. *Pantocsekiella costei* was originally described by Druart & Straub (1988) as *Cyclotella costei* during an ecological and palaeoecological study of a small alkaline, eutrophic lake Paladru (France). *Pantocsekiella costei* (*Cyclotella costei*) is closely related and probably conspecific with *C. comensis* and *C. pseudocomensis* (Kistenich et al., 2014; Duleba et al., 2015). Håkansson & Carter (1990) described *Cyclotella cyclopuncta* from Plitvice Lakes, but later taxonomic analyses of Houk et al. (2010) showed that both species share the same morphological features, and thus, *C. cyclopuncta* was considered a synonym of *P. costei*. This small sized species (4–17 μm in diameter) has been reported from the fossil and modern samples across a range of littoral and pelagic habitats from alkaline, oligo- to mesotrophic lakes (Houk et al., 2010). According to this study it is dominant in oligotrophic karst system, but also present in oligo- to mesotrophic conditions of all other investigated lakes. *P. costei* concurs with *C. comensis* in ecological preference toward oligotrophy, since *C. comensis* was inserted into codon A (Reynolds et al.,

2002; Cellamare et al., 2016). *Lindavia radiosa* has been described from Lake Mondsee (Austria) as *Cyclotella radiosa* (Lemmermann, 1900), but latest taxonomic and phylogenetic analyses resulted with its transfer into genus *Lindavia* based on the position of rimoportula, striae structure, and presence of a complex alveolar structure in the central area (Nakov et al., 2015; Ács et al., 2016). Its biogeographic distribution, mainly in plankton assemblages from subalpine lakes like Mondsee, Staffelsee and Starnberger See, suggests oligo- to mesotrophic ecological preferences (Houk et al., 2010).

Community of Lake Vransko was strongly correlated with conductivity and pH values, constraints during which a small diatom *S. chantaica* remained a common species. Due to the size, *S. chantaica* did not participate in biomass significantly, but it clearly described the phytoplankton assemblage of Lake Vransko. Originally described from Lake Khantajskoe in Siberia (Genkal & Kuzmina, 1985), it is a rare species usually reported from circumpolar sites on the northern hemisphere (Scheffler & Padisák, 2000) and, because of its unclear status, even considered

Figs. 15–18 **15** *Dinobryon sociale* Ehrenberg (Ehrenberg). **16** *Dinobryon divergens* O. E. Imhof. **17** *Dinobryon cylindricum* O. E. Imhof. **18** *Dinobryon sertularia* Ehrenberg; **15, 17** Lake Prošće; **16, 18** Lake Vransko. Scale bars 50 μ m in (15–18)



endangered (Kiss et al., 2012). In terms of ecology, it prefers deep, oligo- to mesotrophic, slightly alkaline lakes (Scheffler & Morabito, 2003). Functional classification places *Stephanocostis* into codon **B**, but due to unusual environmental conditions under which this and other species (e.g., *Aulacoseira baicalensis* (K. I. Meyer) Simonsen) grow, Padisák et al. (2009) proposed raising a separate codon. Based on aforementioned findings, the occurrence of species in karstic systems, and congruous environmental settings of Lake Vransko, we consider *S. chantaica* to fit into codon **A**. Until now, there was no record of this cold-tolerant species from the Mediterranean climate zone.

Cyclotella species from codon **B** have a wide tolerance to various environmental parameters, such as adaptation to high lake stability (Beamud et al., 2015) and low light availability (Reynolds, 1997), allowing them to dominate in more mesotrophic water bodies (Hu et al., 2012). Light deficiency indicated by low Secchi disk depth (Gligora Udovič et al., 2015) and a strong thermal stratification were noted in Lake Visovačko throughout the investigated period, facilitating *P. ocellata* as the main descriptor of the diatom community, with accompanying *Pantocsekiella*

polymorpha and *Discostella pseudostelligera* (Hustedt) Houk & Klee. *Pantocsekiella ocellata* is a diatom described from Lake Balaton, Hungary (Pantocsek, 1901). The latest phylogenetic analyses of the genus *Cyclotella* showed it can be split into several genera and allowed *C. ocellata* to be assigned a type species of a newly described diatom genus, *Pantocsekiella* (Ács et al., 2016). *Pantocsekiella ocellata* is a highly variable species in terms of its morphology and ecology, therefore a number of studies have been performed to delineate its phenotypic plasticity (Kiss et al., 1996; Edlund et al., 2003; Cremer et al., 2005; Genkal & Popovskaya, 2008; Winder et al., 2009; Duleba et al., 2015). *Pantocsekiella ocellata* (*C. ocellata*) is usually considered as a species complex (Edlund et al., 2003) rather than a single species (Duleba et al., 2015). It belongs in a complex together with *Cyclotella krammeri* Håkansson, *Cyclotella rossii* Håkansson, *Cyclotella tripartita* Håkansson, *Cyclotella kuetzingiana* Thwaites, *C. polymorpha* B. Meyer & Håkansson, and *Cyclotella comensis* Grunow (Edlund et al., 2003; Cherepanova et al., 2010; Duleba et al., 2015), with several species transferred into a genus *Pantocsekiella* K. T. Kiss et Ács, gen.

nov.: *P. ocellata*, *Pantocsekiella rossii* (Håkansson) K. T. Kiss et Ács, comb. nov., *Pantocsekiella tripartita* (Håkansson) K. T. Kiss et Ács, comb. nov., *P. polymorpha* (B. Meyer et Håkansson) K.T. Kiss et Ács, comb. nov., and *Pantocsekiella comensis* (Grunow in van Heurck) K. T. Kiss et Ács, comb. nov. (Ács et al., 2016). Besides taxonomic uncertainties of the *C. ocellata* ‘group’, the Visovačko assemblage consisted of classical ‘*ocellata*’ morph, ‘*trichonidea*’ morph with slightly quadrangular contours and transitional forms (Duleba et al., 2015). In the case of Visovačko Lake, population partial sequences of 18S rDNA and rbcL showed no variation in samples with different morphs, and the difference was also low when compared to *C. comensis*, *C. pseudocomensis*, and *C. costei* and suggest that *C. ocellata* and *C. comensis* are two very closely related species that have only recently diverged from one another (Duleba et al., 2015). However, the life-form of *P. ocellata* occurring in chain formation as we observed in the Lake Visovačko has not been previously recorded in literature. Generally, in *Cyclotella* species, colony formation using organic material has been previously well described (Round et al., 1990) and already illustrated by Hustedt (1930). *Cyclotella* colonies can range from more or less amorphous aggregates embedded in mucilage such as in *Lindavia socialis* (in Hustedt 1930 as *C. socialis*) to *C. planctonica* forming wide and rigid gelatinous envelope enclosing the cells in a chain-like formation (Hustedt 1930). The most common *Cyclotella* life forms are the same as we observed in *P. ocellata*, and these include chain colonies of variable length formed by cells interconnected with chitin fibers as in *C. cryptica* (Round et al. 1990) or mucilage material as in *C. quadriuncta*, *C. glomerata*, *C. melosiroides* (Hustedt, 1930), or *C. choctawhatcheeana* (Bosak, pers. obs.).

A range of centric diatoms in lakes Vransko and Visovačko were assorted into groups **A** and **B** attributed with a tolerance to low phosphorus conditions (Tilman & Kilham, 1976; Grigorszky et al., 2006) in stratified oligo- to mesotrophic lake types (Reynolds et al., 2002). Moreover, temperature profiles, mixing pattern, and trophic status of these lakes (Schmidt et al., 2000; Gligora Udovič et al., 2011, 2015) correspond both to the habitat template designated by Reynolds et al. (2002) and Padisák et al. (2009) and to the specified properties of selected species.

Actinocyclus normanii (Gregory ex Greville) Hustedt is a warm stenothermic species usually proliferating during summer. Although it had a very rare occurrence in Lake Visovačko during this study, it has been increasingly reported in the last decades from both lacustrine and riverine systems with different halobity, presumably as result of increased eutrophication, and is considered a potentially invasive species (Kaštovský et al., 2010; Abonyi et al., 2012; Kiss et al., 2012).

Cyclotella-dominated assemblage (Hutchinson, 1967) is favored in a thermally stratified, highly insolated water column (Miracle et al., 1992; Tolotti et al., 2007) with a relatively short retention time and deep mixing (Žutinić et al., 2014). In addition, typology and trophic status of lakes Kozjak and Prošće (Žutinić et al., 2014; Gligora Udovič et al., 2015), higher nutrient (TN and TP) values, lower temperature ranges, and high light conditions promote such diatom composition with no strict delimitation between associations **B** and **C** (Reynolds et al., 2002). Main descriptors of Lake Prošće were codon **C** species *S. neoastreae*, designated as *Stephanodiscus* sp. in previous studies by Žutinić et al. (2014) and Gligora Udovič et al. (2015), and codon **B** species *C. distinguenda* and *C. plitvicensis*. Relatively high biomass and dominance of large sized *S. neoastreae* recorded in Lake Prošće are likely sustained by higher nitrogen supply (Winder et al., 2009) and an indication of increased P concentration (Anneville et al., 2004; Kasperovičienė & Vaikutienė, 2007), although it was noted in oligotrophic systems (Üveges et al., 2012). *Stephanodiscus neoastreae* was described from Bornhoveder See (Germany) by Håkansson & Hickel (1986) and is easily distinguished from other similar species (e.g., *S. rotula* (Kützing) Hendey, *S. niagarae* Ehrenberg) by the absence of valve face fulcportula, an important morphological character for identification of *Stephanodiscus* taxa. On the other hand, *S. minutulus* (Kützing) Cleve & Möller and *S. parvus* Stoermer & Håkansson can be easily confused because of their small size. Although both species can co-occur in various aquatic ecosystems usually characterized by elevated ion concentrations (Krammer & Lange-Bertalot, 1991), they can be separated by the position of the valve face fulcportula (see Figs. 6, 7, 8, 9; 139–150 in Håkansson, 2002).

In compliance with its trophic status, Lake Kozjak was characterized by a codon **B** centric *C.*

distinguenda, a widespread species characteristic for oligo- to mesotrophic lakes (Wunsam et al., 1995). It may be erroneously classified as *Lindavia radiosa* due to overlapping size and other morphological characters indiscernible under the light microscope (Žutinić et al., 2014; Gligora Udovič et al., 2015). *C. distinguenda* is closely related and morphologically similar to *C. plitvicensis* (e.g., diameter range and striae density; (Przybyłowska-Lange, 1990; Huber, 2009), a species with highly restricted ecological niche. Although *C. plitvicensis* was not confirmed in Kozjak during this research, it was noted in lakes Prošće and Visovačko and placed accordingly into codon **B**. *Stephanodiscus*, a reliable indicator of more eutrophic conditions (Kilham et al., 1986; Hall & Smol, 1992), was represented by *S. minutulus* (codon **B**), *S. neoastrea* and *S. parvus* (codon **C**) in both lakes, although with substantially lower biomass in Lake Kozjak. *Cyclotella distinguenda* and the regionally endemic *C. plitvicensis* are similar in morphology, but nonetheless, both species can be separated by the shape of the central area (transversally undulated in *C. distinguenda* vs. flat in *C. plitvicensis*) and the number of rimoportulae (at least one in *C. distinguenda* and one in *C. plitvicensis*). Latest phylogenetic analyses suggest both species should remain in the genus *Cyclotella* based on the characteristic position and shape of the rimoportula, as well as the presence of striae with equal length (Ács et al., 2016).

Alongside diatoms, chrysophytes frequently constitute a significant component of phytoplankton assemblages of karst lakes (Srdoč et al., 1992; Morata et al., 2003; Gligora et al., 2007; Žutinić et al., 2014; Gligora Udovič et al., 2015). The major representative, *Dinobryon* spp. complex, is assorted into functional group **E** (Reynolds et al., 2002; Padisák et al., 2009). Since codon **E** is designated for small, shallow, base poor lakes, or heterotrophic ponds, current habitat description does not fit into a description of typical karst lakes. Taxa belonging to group **E** are known to dominate spring-early summer plankton in oligotrophic–mesotrophic lakes (Žutinić et al., 2014), because they are mixotrophs and can supplement nutrient uptake by the phagotrophic ingestion of bacteria (Reynolds et al., 2002; Kamjunke et al., 2007). Studies addressing the rate of phagotrophy of *Dinobryon* in various environmental conditions showed contrasting results. *Dinobryon cylindricum*,

often a dominant species in Croatian karst lakes, efficiently utilized phagotrophy in natural lakes (Laybourn-Parry & Marshall, 2003), but appeared rather inadequate when in culture environment (Caron et al., 1993). Olrik (1998) showed that Chrysophytes used bacteria more as a P substitute, than for C uptake. As supported by the mentioned studies, *Dinobryon* complex in karst lakes can be attributed to codon **E** if interpreted in the context of distinctive conditions prevailing in such ecosystems. Since no studies on the feeding preferences of *Dinobryon* were conducted in Croatian or similar karst lakes, these findings rise up unanswered questions that should be clarified by further investigations. Besides *D. cylindricum*, other regularly occurring species in Croatian karst lakes, like *D. divergens*, *D. sociale*, and *D. sertularia* Ehrenberg, all show similar ecological preferences with their simultaneous appearance. Besides centric diatoms and representatives of codon **E**, dinoflagellates belonging to the functional group **L_O** often tend to appear in large abundance, as well as biomass, thus defining phytoplankton community in the habitat (Gligora Udovič et al., 2015). The features they share with genus *Dinobryon* include mixotrophy and motility (Popovsky & Pfister, 1990). Codon **L_O** covers a wide range of habitats comprising deep and shallow, oligo- to eutrophic, medium-to-large lakes. Based on ecological traits of its representative dinoflagellate species, group **L_O** defines phytoplankton assemblages in the studied lakes and supports a concept of **A–B** and **E–L_O** co-occurrence as suggested by Gligora Udovič et al. (2015) and herewith, can serve as a descriptor of natural communities in deep oligo-mesotrophic lakes of Dinaric karst.

Several representatives for diatom-based Reynolds FGs of four lakes in Croatia were described in this paper: *P. costei* and *S. chantaica* for group **A**, *P. ocellata* and *C. distinguenda* for group **B**, and *S. neoastrea* for group **C**, together with their ecological preferences which clearly correspond to pertaining functional classification. Selected and coexisting functional groups define natural, oligo- to mesotrophic karst deep lake systems. Every anthropogenic impact is clearly shown on these pristine ecosystems, therefore increased knowledge about keystone phytoplankton species in populations is crucial in order to understand and predict the response of environment to shifting conditions.

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